A Genome-wide Association Meta-analysis of Preschool Internalizing Problems

Kelly S. Benke, PhD, Michel G. Nivard, BA, Fleur P. Velders, PhD, Raymond K. Walters, PhD, Irene Pappa, MD, Paul A. Scheet, PhD, Xiangjun Xiao, MS, Erik A. Ehli, PhD, Lyle J. Palmer, PhD, Andrew J.O. Whitehouse, PhD, Frank C. Verhulst, MD, PhD, Vincent W. Jaddoe, MD, PhD, Fernando Rivadeneira, MD, PhD, Maria M. Groen-Blokhuis, MD, Catharina E.M. van Beijsterveldt, PhD, Gareth E. Davies, PhD, James J. Hudziak, MD, Gitta H. Lubke, PhD, Dorret I. Boomsma, PhD, Craig E. Pennell, MD, PhD, Henning Tiemeier, PhD, MD, Christel M. Middeldorp, MD, PhD, for the Early Genetics and Lifecourse Epidemiology Consortium

Objective: Preschool internalizing problems (INT) are highly heritable and moderately genetically stable from childhood into adulthood. Gene-finding studies are scarce. In this study, the influence of genome-wide measured single nucleotide polymorphisms (SNPs) was investigated in 3 cohorts (total N = 4,596 children) in which INT was assessed with the same instrument, the Child Behavior Checklist (CBCL). **Method:** First, genome-wide association (GWA) results were used for density estimation and genome-wide complex trait analysis (GCTA) to calculate the variance explained by all SNPs. Next, a fixed-effect inverse variance meta-analysis of the 3 GWA analyses was carried out. Finally, the overlap in results with prior GWA studies of childhood and adulthood psychiatric disorders and treatment responses was tested by examining whether SNPs associated with these traits jointly showed a significant signal for INT. **Results:** Genome-wide SNPs explained 13% to 43% of the total variance. This indicates that the genetic architecture of INT mirrors the polygenic model underlying adult psychiatric traits. The meta-analysis did not yield a genome-wide significant signal but was suggestive for the PCSK2 gene located on chromosome 20p12.1. SNPs associated with other psychiatric disorders appeared to be enriched for signals with INT ($\lambda = 1.26$, $p < .03$). **Conclusion:** Our study provides evidence that INT is influenced by many common genetic variants, each with a very small effect, and that, even as early as age 3, genetic variants influencing INT overlap with variants that play a role in childhood and adulthood psychiatric disorders. J. Am. Acad. Child Adolesc. Psychiatry, 2014;53(6):667–676. **Key Words:** GWA study, internalizing problems, pcsk2, variance explained, GCTA

Preschool internalizing problems (INT) are relatively prevalent, often not self-limited, and associated with significant morbidity. A recent study investigating prevalence rates of *DSM-IV* disorders in a sample of 2,475 Norwegian 4-year-olds found, for example, that 1.5% of the children fulfilled the criteria for any anxiety disorder and 2% for a depressive disorder.[1] Preschool INT are persistent into childhood, as shown by several longitudinal studies.[2-5]

Supplemental material cited in this article is available online.

Twin studies have shown a substantial influence of genetic factors on preschool INT. Heritability estimates of INT, assessed across a range of instruments, are mostly around 40% and 50% (range, 36%–75%), with study samples varying from 822 to 6,783 twin pairs.[5-9] These heritability estimates are similar to or even higher than the estimates found for anxiety and depressive symptoms and disorders in adults.[10,11] Moreover, genetic factors influencing INT at age 3 years continue to have an effect later in life, even into adulthood (M.G. Nivard, C.V. Dolan, K.S. Kendler, K.J. Kan, G. Willemsen, C.E.M. Van Beijsterveldt , R.J.L. Lindauer, J.H.D.A. Van Beek, L.M. Geels, M. Bartels,

C.M. Middeldorp, D.I. Boomsma, unpublished material, April 2013).[5]

There are numerous gene-finding studies for anxiety and depression in adults, but gene-finding studies on childhood INT are scarce. There has been only 1 genome-wide association (GWA) study that analyzed anxiety-related behaviors in children (N = 2,810) 7 years old.[12] None of the effects of the top 10 single nucleotide polymorphisms (SNPs) (p values between 8.7×10^{-7} and 1.2×10^{-4}) were replicated in an independent sample of 4,804 children. In addition, a genome-wide complex trait analysis (GCTA) was performed in the discovery sample.[13] Such an analysis does not focus on the effect of each SNP separately, but calculates the variance explained by all genome-wide SNPs. For anxiety-related behaviors, the GCTA-yielded estimates were between 0.01 (standard error [SE] = 0.11) and 0.19 (SE = 0.12). The authors concluded that common SNPs do not explain as much of the genetic influence on anxiety at age 7 as on other psychiatric phenotypes.

We present a genome-wide approach to investigate the etiology of preschool INT. Genome-wide SNP data were analyzed from 3 cohorts with a total of 4,596 children in which INT was measured with the same instrument. Each cohort carried out a genome-wide association study (GWAS). These results were, first, used to estimate the variance attributable to all SNPs in each cohort. A meta-analysis of the results of the 3 GWAS was performed next, aiming to identify genetic variants influencing INT. Finally, overlap between our meta-analysis results and results from prior GWA (meta-)analysis studies was investigated. We analyzed whether SNPs associated with a range of psychiatric disorders jointly yielded a significant signal in the meta-analysis results of INT at age 3 years. We have not restricted these analyses to SNPs associated with internalizing disorders (depression), but have also analyzed SNPs associated with disorders usually diagnosed in childhood or psychotic disorders. There is frequent co-morbidity between childhood internalizing disorders and other disorders in childhood, and internalizing symptoms predict a range of disorders in adulthood, including disruptive disorders and schizophrenia.[1,14-16] This could well be due to overlapping genetic risk factors. Furthermore, it has been suggested that treatment-resistant depression is influenced by specific risk factors including early age of onset,[17] which may signify that disorders resistant to various treatments bear a unique genetic signature. Although literature does not provide a direct link to internalizing problems in children and treatment response in adults, we wished to explore whether treatment-resistant SNPs were enriched in preschool internalizing children. Therefore, SNPs were also selected from GWAS of treatment response in adults.

METHOD

Participants

Participants were recruited from 3 large population-based studies (Table 1).

Generation R. The Generation R study (www.generationr.nl) is a prospective population-based cohort of 9,745 children born in Rotterdam, the Netherlands, whose due dates were between April 2002 and January 2006.[18,19] Data from a total of 7,893 children were available and eligible for follow-up. DNA was extracted from cord blood taken at birth. Children of Northern European descent, as determined by

TABLE 1 Description of Cohorts, Internalizing Problem (INT) Scores and Measure, and Estimates of Variance Explained by All Single Nucleotide Polymorphisms (SNPs) Obtained With Density Estimation Method (DE) and With Genetic Complex Trait Analysis (GCTA)

Characteristic	Generation R		NTR		Raine	
N (% girls)	2,037	(49)	1,475	(50)	1,084	(49)
Mean age (SD)	3.0	(0.10)	3.31	(0.26)	2.2	(0.15)
Mean INT score (SD)	4.0	(3.4)	7.8	(6.0)	7.2	(5.1)
INT score range	0,23		0,33		0,37	
CBCL version	CBCL/1½ - 5		CBCL/2-3		CBCL/2-3	
Website	www.generationr.nl		www.tweelingenregister.org/en/		www.rainestudy.org.au	
Explained variance by all SNPs in %						
DE (p value)	41	(0.04)	31	(0.41)	43	(0.58)
GCTA (p value)	26	(0.07)	18	(0.30)	13	(0.33)

Note: CBCL = Child Behavior Checklist; NTR = Netherlands Twin Registry.

principal component analyses of GWA data, were selected.[20] Of 5,908 children with DNA available, 2,841 children of Northern European descent were identified, of whom 2,037 children (50% girls) had SNP data and the INT score available. The Medical Ethical Committee of the Erasmus Medical Centre, Rotterdam (MEC 217.595/2002/20) approved the study protocol, and participants gave informed consent in writing.

Netherlands Twin Register. The Netherlands Twin Register (NTR) (www.tweelinGenerationRegister.org) is a prospective study involving families with twins. The NTR was established at the VU University Amsterdam in 1987 and includes twins born from 1986 onward.[21] Data collection is ongoing. Parental ratings of problem behavior are available for ages 2, 3, 5, 7, 10, and 12 years. Subsamples of young twins were invited to participate in experimental and laboratory studies and to provide a DNA sample, either by whole blood or by buccal swabs.[22] Dizygotic (DZ) twin pairs were included in the analyses while correcting for the dependence between their measures. From monozygotic (MZ) twin pairs, 1 twin was randomly selected. This yielded a sample of 1,475 children (50% girls), belonging to 1,031 families, with genotype and phenotype data. The study was approved by the Central Ethics Committee on Research involving human participants of the VU University Medical Centre, Amsterdam, and an institutional review board (IRB) certified by the U.S. Office of Human Research Protections (IRB number IRB-2991 under Federal-wide Assurance-3703; IRB/institute codes, NTR 03-180).

Western Australian Pregnancy (Raine) Cohort Study. The Western Australian Pregnancy (Raine) Cohort Study (www.rainestudy.org.au) is a prospective cohort representative of those presenting to an antenatal tertiary referral center in Western Australia.[23,24] There were 2,900 pregnant women recruited between 1989 and 1991 as part of a randomized control trial to investigate the association of repeated ultrasound measurements during pregnancy on birth outcomes. DNA was collected from blood at the 14 year follow-up. There are 1,084 children (49% girls) for whom DNA and INT data at 2 to 3 years of age are available. Participant recruitment and all follow-ups of their families were approved by the Human Ethics Committee at King Edward Memorial Hospital and/or Princess Margaret Hospital for Children in Perth.

Genotyping, Quality Control, and Imputation Procedures

DNA was extracted from blood in Raine and Generation R and from buccal swabs (n = 1,087) and whole blood (n = 388) in NTR. Excellent concordance between SNP genotyped in buccal and blood samples has been shown in 331 individuals for whom both kinds of samples were available.[25] For Raine and Generation R, genotyping was performed on Illumina platforms. For NTR, the affymetrix 6.0 platform was used. Table S1, available online, provides details of the genotyping centers, platforms, calling software, analytic and imputation software, and details of the preimputation and postimputation filtering criteria per study. Basic quality checks for each SNP included call rates and Hardy-Weinberg statistics. For Generation R and Raine, each sample was checked for excess heterozygosity, gender accuracy, relatedness (identity by descent), and missingness. NTR samples were also checked for incorrect Mendelian inheritance patterns, as parents of twins were genotyped in 25% of families. After prefiltering, phased genotype data were imputed to build 36 (release 22) of the original HapMap CEU reference panel for NTR and Raine studies, and the consensus panel for the Generation R study resulting in 2.5 to 3 million SNPs for GWA analysis.[26]

Measurement of Internalizing Problems

In all cohorts, INT was assessed by the internalizing problem scale of the Child Behavior Checklist (CBCL). In the most recent version of the CBCL 1½ – 5, the INT scale consists of 36 items. Of these items, 34 were measured in all 3 cohorts and were, therefore, used to obtain the INT sum score for the analyses. Example items are "Acts too young for age," "Worries a lot," and "Clings to adults or too dependent." For each item, the rater must select a score of 0 (not true), 1 (somewhat or sometimes true), or 2 (very true or often true), resulting in a potential score range of 0 to 68. Table S2, available online, provides a list of the 34 items and their corresponding subscales. In Generation R and Raine, the primary caregiver, usually the mother, filled out the questionnaire. These studies thus included paternal and other caregiver raters, although they were rare (5% in Generation R and data not available in Raine). In NTR, only maternal ratings were analyzed.

Statistical Analysis

GWA-based Analysis Within Cohorts. Each cohort modeled the outcome as the square root of the sum score, which was chosen based on simulation and regression diagnostics (details are provided in the Supplement, available online). The significance of the SNPs was tested in the following regression equation:

$$\sqrt{\text{INT}} = \alpha_0 + \beta_1\text{SNP} + \beta_2\text{PC} + \beta_3\text{Sex}$$

SNP was coded as 0, 1, and 2, reflecting the number of alternate alleles for a given individual. To correct for population stratification, 10 principal components (PC) were included as covariates in the NTR GWA, and 2 were included in Raine and Generation R. The Generation R and NTR studies did not adjust for age, given the age at time of data collection was restricted to 3 years or younger; the Raine study accepted children 2 to 3 years of age, and adjusted for age. In Generation R and Raine, the analyses were performed in mach2qtl software.[27,28] In NTR, the analyses were performed in PLINK[29] using the option "—family" to account for the dependence between INT measured in DZ twins from the NTR.

Variance Explained by All SNPs. The joint effect of all SNPs in explaining the variance of INT was calculated in each cohort using the density estimation method (DE) proposed by So *et al.*[30] and using GCTA. In GCTA, a genetic relationship matrix is calculated, based on all SNPs, reflecting the genetic similarity in unrelated individuals. Next, the variance explained by these SNPs is estimated in a linear mixed model in which the measure of the genetic similarity is included as a random effect to predict the phenotype.[13] The analyses were corrected for the covariates included in the GWA analyses. Moreover, related individuals were excluded (threshold = 0.025).

In contrast to GCTA, DE does not use measured SNPs, but uses the *z*-transformed t statistics of the regression coefficients as obtained in a GWA analysis to estimate the explained variance. As it is more common to provide GWA results to a consortium than genotype data, this method is more suitable when using data from several cohorts. The basic idea is to compare the distribution of *z*-transformed t statistics of the regression coefficients of genome-wide SNPs to the theoretical null distribution of *z* statistics representing no effects. Deviation of the observed statistics from the theoretical null distribution indicates that SNPs explain part of the variance. Specifically, the observed *z* statistics, which contain error due to sampling fluctuation, are first corrected to obtain *z* statistics representing "true" effect sizes. The *z* statistics can then be combined by summing the contributions of all SNPs using sums of squares as in analysis of variance. These sums of squares are computed based on the estimated effect sizes and the study sample size, as well as the number of included covariates and their joint effect size. The resulting sum is an estimate of the total proportion of phenotypic variance explained by the SNPs in the analysis. Before applying DE, we carried out linkage disequilibrium (LD) pruning as suggested by So *et al.*[30] using HapMap CEU genotypes as a reference set, which had been used for imputation in all 3 cohorts.

The *p* values for the variance-explained estimates are calculated using Monte Carlo *p* values. This is necessary because the sampling distribution for the estimates is skewed and biased, and standard errors are potentially misleading. Briefly, in each of 4,000 Monte Carlo replications, variance explained is estimated using simulated t statistics for each SNP under the null hypothesis (i.e., 0 variance explained). Then the estimated *p* value for the observed data is the proportion of Monte Carlo replications with estimated variance explained greater than or equal to the estimate for the observed data. (Simulation results showing that this method is appropriate for the variance-explained estimates are available on request from one of the authors (R.K.W.).

Meta-analysis. An inverse variance meta-analysis was performed in METAL.[31] Comparing a fixed effect analysis with a random effect analysis did not show different results. Therefore, we report the findings from the fixed effect model. Because the genomic control λ (the median χ^2 association statistic divided by the median expected under the null) within each cohort were close to 1.0 and thus indicated no evidence for inflation, we applied a genomic control solely at the meta-analysis stage. We only considered SNPs whose minor allele frequency was greater than 0.01. We also applied quality filtering, requiring that the imputed SNPs had a quality score above 0.30 for the Generation R and Raine studies, and a PLINK info score between 0.80 and 1.1 for the NTR study. We considered any SNPs with a *p* value of less than 5×10^{-8} to achieve genome-wide statistical significance.

Analysis of SNPs Associated With Psychiatric Disorders and Treatment Response. We conducted an online search (www.genome.gov/gwastudies) in January 2013 for SNPs with a *p* value of less than 1×10^{-5} in GWAS of

FIGURE 1 Manhattan plot for the genome-wide association meta-analysis of preschool internalizing problems (INT) across 3 cohorts. Note: Chromosome is displayed on the x-axis and the association statistic, expressed as –log10(*p* value), is given on the y-axis. Points that fall within the black (–log10(5×10^{-8}) and gray lines (–log10(1×10^{-5}) are signals suggestive for association. Pink diamonds denote the single nucleotide polymorphisms (SNPs) of interest on chromosomes 9 and 20.

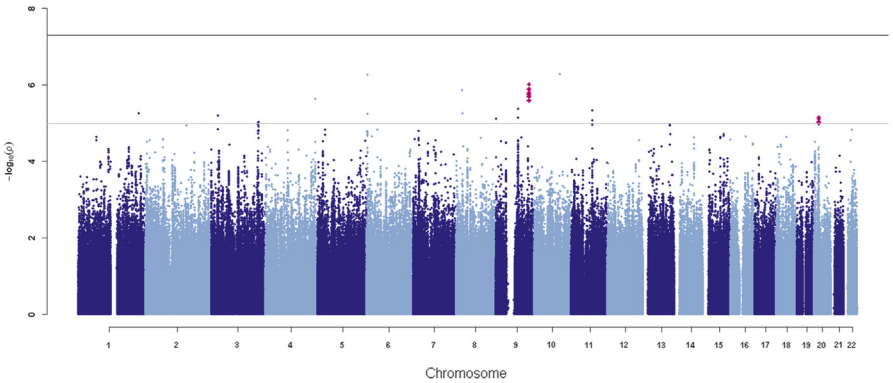


TABLE 2 The 30 Genetic Variants Most Strongly Associated With Preschool Internalizing Problems (INT) in the Genome-wide Association (GWA) Meta-analysis

Chr Region	SNP	Position	MAF	Ref Allele	Direction	β	(95% CI)	*p* Value
1q42.12	rs360059	224143755	0.3564	A	−−−	−0.11	(−0.15, −0.061)	5.5810^{-6}
1q42.12	rs1223245	224149048	0.3578	A	+++	0.11	(0.061, 0.15)	5.4410^{-6}
3q26.31	rs4894527	173424920	0.2921	T	+++	0.11	(0.060, 0.16)	9.8210^{-6}
3q26.31	rs1878007	174281494	0.1702	A	−−−	−0.12	(−0.18, −0.069)	9.3410^{-6}
4q35.1	rs10013166	183714323	0.4416	A	+++	0.11	(0.062, 0.15)	2.3610^{-6}
5q33.1	rs1062177	151164894	0.2475	T	−?−	−0.16	(−0.23, −0.091)	8.2610^{-6}
6p25.2	rs2272990	3022140	0.0823	T	?−−	−0.39	(−0.55,− 0.24)	5.3910^{-7}
6p25.2	rs9405191	3031952	0.153	C	?−−	−0.29	(−0.42, −0.17)	5.7510^{-6}
6p25.2	rs9391981	3032005	0.1526	C	?−−	−0.29	(−0.42, −0.17)	5.7510^{-6}
8p21.3	rs6557600	22819193	0.2273	A	−−−	−0.13	(−0.18, −0.076)	1.3710^{-6}
8p21.3	rs310272	23699434	0.3718	C	+++	0.10	(0.060, 0.15)	5.5210^{-6}
9p24.3	rs12000567	779499	0.0165	A	?++	0.63	(0.35, 0.91)	7.7510^{-6}
9q21.31	rs17266958	82455069	0.0756	T	−−−	−0.20	(−0.28, −0.11)	4.2010^{-6}
9q21.31	rs17083743	82481285	0.071	A	+++	0.20	(0.11, 0.29)	7.2110^{-6}
9q33.1	rs10818415	121811389	0.1769	T	−−−	−0.14	(−0.12, −0.081)	1.7810^{-6}
9q33.1	rs10984795	121814167	0.1853	A	+++	0.14	(0.082, 0.20)	1.6110^{-6}
9q33.1	rs16909317	121814340	0.1849	T	−−−	−0.14	(−0.19, −0.081)	2.0010^{-6}
9q33.1	rs2416740	121817157	0.1842	A	−−−	−0.14	(−0.19, −0.079)	2.5610^{-6}
9q33.1	rs2416741	121817280	0.1835	A	−−−	−0.14	(−0.20,− 0.081)	1.7510^{-6}
9q33.1	rs10984803	121820192	0.1749	A	−−−	−0.14	(−0.20, −0.084)	1.2410^{-6}
9q33.1	rs10818418	121820770	0.1841	A	+++	0.14	(0.083, 0.20)	1.3410^{-6}
9q33.1	rs2416745	121821075	0.1766	T	+++	0.14	(0.084, 0.20)	9.5110^{-7}
9q33.1	rs10984807	121822291	0.188	T	+++	0.14	(0.082, 0.20)	2.5310^{-6}
10q23.33	rs640090	95382575	0.0715	C	?++	0.55	(0.33, 0.76)	5.2410^{-7}
11q14.1	rs12270115	79162785	0.1334	A	+++	0.15	(0.088, 0.22)	4.6610^{-6}
11q14.1	rs12287037	79205014	0.1918	T	+++	0.12	(0.068, 0.17)	8.4610^{-6}
20p12.1	rs2281204	17364812	0.3416	A	+++	0.10	(0.056, 0.14)	9.7710^{-6}
20p12.1	rs890609	17365013	0.3411	A	−−−	−0.10	(−0.14, −0.056)	9.9410^{-6}
20p12.1	rs2021786	17369978	0.3356	T	−−−	−0.10	(−0.15, −0.058)	6.9510^{-6}
20p12.1	rs2021785	17370063	0.3356	T	−−−	−0.10	(−0.15, −0.058)	6.9810^{-6}
20p12.1	rs13039651	17371040	0.3359	T	−−−	−0.10	(−0.15, −0.057)	7.6710^{-6}
20p12.1	rs2269020	17375229	0.3442	C	−−−	−0.10	(−0.15, −0.056)	9.1610^{-6}

Note: Chr Region = chromosomal region; MAF = minor allele frequency; NTR = Netherlands Twin Registry; SNP = single nucleotide polymorphisms. A direction is provided for each study in the following order: NTR, GenR, and Raine. Plus indicates that the β for the association between the SNP and square root of the Internalizing Problem score is positive. Minus indicates a negative association. Question mark indicates that the SNP did not survive the postimputation QC process for that study.

psychiatric disorders and treatment response performed in children or adults. We first analyzed SNPs associated with internalizing disorders (major depression). Next, in 3 steps, SNPS were added that were associated with disorders usually diagnosed in childhood (attention-deficit/hyperactivity disorder [ADHD], conduct disorder, and autism) and psychotic disorders (bipolar disorder and schizophrenia), that were associated with treatment response for antidepressants, lithium, and antipsychotics and that were located in candidate genes for major depression based on hypotheses regarding the etiology (derived from Table 1 in Bosker *et al.*[32]).

To determine whether these candidate SNPs were associated beyond expectation under the null in our meta-analysis, we calculated the genomic control λ. Next, a null distribution of λ is created by sampling 10,000 sets of *p* values equal in size to the set of SNPs being tested. The observed λ in each of the 4 sets is significant when it exceeds the λ in the null distribution.

RESULTS

Variance Explained by All SNPs

In the Raine and NTR studies, the GWA analysis was performed on 2,543,887 autosomal SNPs imputed from the HapMap original panel that passed initial quality control measures (Table S1, available online). In Generation R, a total of 3,021,329 autosomal SNPs imputed from the HapMap consensus panel passed initial quality control measures and were analyzed in the GWA. QQ and Manhattan plots for each cohort are

provided in Figures S1 and S2, available online. The genomic control λ was very close to 1 ($\lambda_{GC} = 1.02$), indicating that there was no evidence for inflated test statistics.

Next, DE analyses and GCTA were performed (Table 1). After pruning for LD, totals of 29,588, 29,612, and 42,800 SNPs were used in the DE analyses of NTR, Raine, and Generation R, respectively. The variance explained by these pruned SNPs varied between 31% and 43%. The variance explained by covariates in each analysis was negligible. In GCTA, the variance explained varied between 13% and 26%. In Generation R, the estimate based on DE was significant ($p = 0.04$) and the GCTA estimate approached significance ($p = 0.07$). The findings were not significant for NTR and Raine. Given a heritability estimate of 59% for INT at age 3 years,[5] this signifies that the SNPs capture between 22% and 72% (13%/59% and 43%/59%, respectively) of the genetic variance.

Genome-wide Association Analyses, Quality Control, and Meta-analysis

After applying post-GWA control measures, there were 2,403,520 SNPs present in both the consensus and the original HapMap panels included in the meta-analysis. At a threshold of $p \leq 5 \times 10^{-8}$, no genome-wide significant findings were detected for the meta-analyzed results, as shown in the Manhattan plot (Figure 1). Table 2 lists the top 30 SNP for which the p values were smaller than 1×10^{-5}, which we considered to be potentially suggestive. Interesting signals that contained multiple suggestive SNPs in an independent region from chromosome 9q33.1 and chromosome 20p12.1 were apparent, and regional plots are shown in Figures S3A and S3B, available online. The 9q33.1 region is an intergenic region and is not in LD with the nearest gene upstream (DBC1 implicated in bladder cancer) or downstream (CDK5 genes implicated in rheumatoid arthritis). The current ENCODE annotations do list 2 interesting nearby noncoding RNAs (lincRNAs) in this region: RP11-360A18.2 and RP11-360A18.1 (Ensembl version ENSG0000026 1432.1 and ENSG00000225960.1,respectively), but the SNPs located near these coordinates are also not in strong LD with our region. The 20p12.1 signal, however, appeared to be in the PCSK2 gene, as indicated in the regional plots provided in Figures S3A and S3B, available online.

Analysis of SNPs Associated With Psychiatric Disorders and Treatment Response

Table 3 shows the results of the analyses of the joint effect of the 4 sets of SNPs with a p value of less than 1×10^{-5} in GWAS of psychiatric disorders and treatment response as found online (at www.genome.gov/gwastudies). The GWAS of internalizing disorders (depression), psychotic disorders (schizophrenia and bipolar disorder), and treatment response were performed in adults. For the GWAS of the disorders usually diagnosed in childhood, both adult and children's samples were used. The SNPs associated only with internalizing disorders did not show a λ significantly greater than 1.0. Adding SNPs associated with disorders usually diagnosed in childhood and with psychotic disorders yielded a significant λ of 1.26. The addition of SNPs associated with treatment response for antidepressants, lithium, and

TABLE 3 λ of the Analysis of the Joint Effect on Preschool Internalizing Problems (INT) of Genetic Variants With *p* Values of 1×10^{-5} in Prior Genome-wide Association Studies for Internalizing Disorders (Depression), Psychotic Disorders (Bipolar Disorder and Schizophrenia), Disorders Usually Diagnosed in Childhood (Attention-Deficit/Hyperactivity Disorder, Autism, and Conduct Disorder), Treatment Response, and Genetic Variants Located in Candidate Genes for Major Depressive Disorder (MDD)

Selected SNPs	No. SNPs in Database	No. SNPs in Filtered Meta-analysis Results	λ	Bootstrapped *p* Value
Internalizing disorders	61	59	1.18	0.29
Internalizing disorder, psychotic disorders and disorders usually diagnosed in childhood	368	320	1.26	0.03
Internalizing disorders, psychotic disorders, disorders usually diagnosed in childhood, and treatment response on antidepressants, lithium, and antipsychotics	472	412	1.17	0.08
≥3 SNPs in candidate genes for MDD	594	472	1.20	0.04

Note: SNP = single nucleotide polymorphisms.

antipsychotics led to a worsening of the signal. However, adding SNPs in candidate genes for major depressive disorder (MDD) (Table 1 in Bosker *et al.*[32]) made the λ significant again, at 1.20. Figure 2 shows the QQ plot of the 320 SNPs with the largest λ of 1.26. A full list of the SNPs is available on request from the first author.

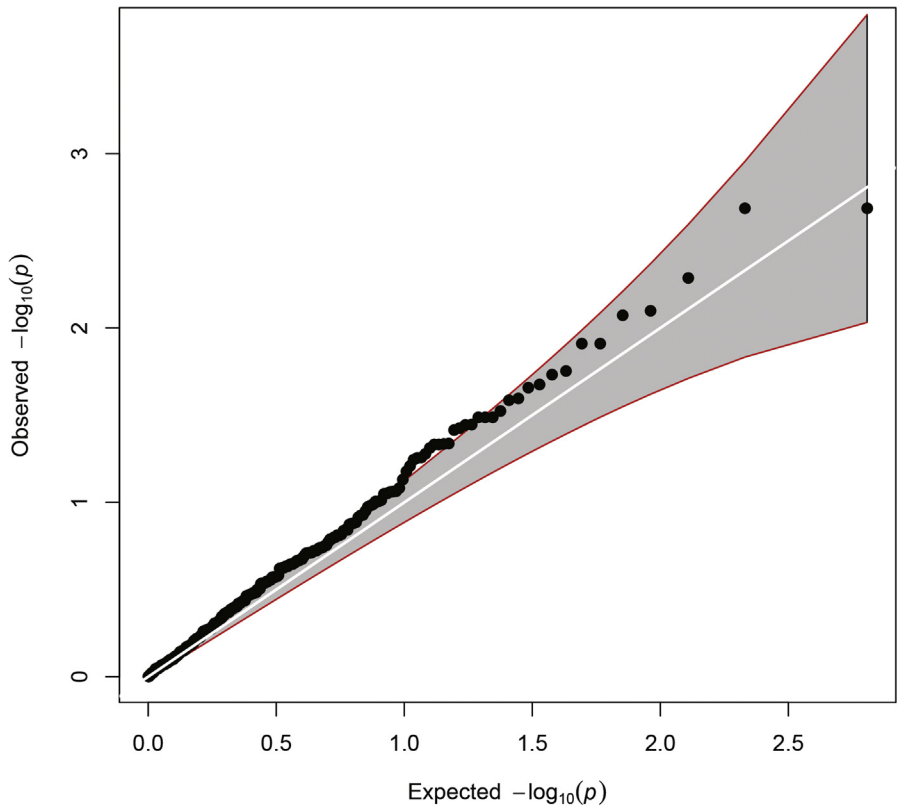
None of the above-mentioned investigated SNPs were localized in the 20p12.1 or the 9q33.1 regions that we highlighted from the results in our GWA meta-analysis. The minimum p value from these SNPs was 0.002 and did not meet criteria for a suggestive finding.

DISCUSSION

Genome-wide SNP data were used to investigate genetic factors influencing INT. Several findings were noteworthy. First, the analysis of variance explained by all SNPs indicated that the common variation as measured with SNPs explains at least 22% of the genetic variance. Second, the meta-analysis of the results of the GWA analyses in the 3 cohorts did not yield a genome-wide significant effect, but 2 areas showed suggestive findings. Third, SNPs that were suggestively associated with childhood and adult psychiatric disorders in prior GWAS or candidate gene studies for MDD showed a joint significant effect on INT at age 3 years, although none of these SNPs individually reached the level of suggestive significance ($p < 1 \times 10^{-5}$).

We note, regarding our first result, that the estimates vary quite widely (between 13% and 43% of the total phenotypic variance is explained), and that, for 2 of the 3 cohorts, the estimates of the variance explained by all SNPs were not significant. The differences in estimates seem mostly due to the method: GCTA estimates were consistently lower than DE estimates. The lack of significance in Raine and NTR appears to be a matter of sample size, given that, within 1 method, the estimates are rather similar for the 3 cohorts. Focusing on the (nearly) significant results for Generation R suggests that between 44% and 69% of the genetic variance is explained

FIGURE 2 Quantile–quantile plot for the joint effect on preschool internalizing problems (INT) of single nucleotide polymorphisms (SNPs) with p values less than 1×10^{-5} in prior genome-wide association meta-analyses of childhood and adulthood psychiatric disorders and treatment responses. Note: Gray-shaded area represents 95% CI and white line represents equality between observed and expected −log10(p values). The λ value of 1.26 is significant ($p < .001$).



by common SNPs. Together with the significant joint effect of SNPs associated with other psychiatric phenotypes, these outcomes point to the conclusion that INT at age 3 years is influenced by a large number of genetic variants, each with a small effect. Moreover, even INT measured as early as age 3 years genetically overlaps with adult psychiatric phenotypes.

These results add to the picture as painted by Visscher *et al.*,[33] reviewing 5 years of GWA studies. Complex phenotypes, psychiatric or somatic, seem to be highly polygenic, and genetic variants can influence multiple traits; that is, there is pleiotropy. Specifically focusing on psychiatric phenotypes, a polygenic architecture has been suggested for adult anxiety disorders, major depression, schizophrenia, and bipolar disorder, with GCTA estimates of around one-third to one-half of the genetic variance explained by common SNPs.[34-38] Pleiotropy has been detected for ADHD, autism and schizophrenia, bipolar disorder, and major depression in studies using genome-wide SNP or copy number variant data.[39,40] Twin studies have also shown substantial genetic overlap within internalizing symptoms or disorders[41,42] and between internalizing disorders and mania and schizophrenia,[43,44] in addition to stable genetic influences from childhood into adulthood for anxious depression and attention problems (M.G. Nivard, C.V. Dolan, K.S. Kendler, K.J. Kan, G. Willemsen, C.E.M. Van Beijsterveldt, R.J.L. Lindauer, J.H.D.A. Van Beek, L.M. Geels, M. Bartels, C.M. Middeldorp, D.I. Boomsma, unpublished material, April 2013).[45]

Although the sample had 80% power to detect an effect explaining approximately 0.90% of the variance[46] and approximately 70% to detect an effect explaining 0.25% of the variance (Table S3, available online), and although the INT measure was similar in the 3 cohorts and assessed at the same age, in the meta-analysis, there were no SNPs with a genome-wide significant effect. Given the DE and GCTA results, this indicates that sample size was still insufficient for what were apparently very small effect sizes. This is in agreement with the results for a continuous phenotype such as height, in which a GWA analysis in more than 180,000 individuals detected hundreds of genetic variants, explaining jointly 10% of the phenotypic variance.[47]

In 2 regions, there were SNPs with a p value of less than 1×10^{-5} in the meta-analysis. One is located in an intergenic region at chromosome 9. According to the latest results from ENCODE, this is also not a regulatory region of the genome.[48-50] The other region was at chromosome 20 and included SNPs of the PCSK2 gene. PCSK2 is an important protein in the processing of proinsulin to insulin,[51] and PCSK2 variants have been correlated with insulin resistance,[52,53] myocardial infarction,[54] and age at menarche.[55] The link between depression and cardiovascular disease has long been recognized.

We compared our results with the results from the GWA analysis of anxiety-related behaviors in 7-year olds.[13] Similar to the investigators' own replication effort, we did not find significant effects for their reported top SNPs. The lowest p value in the current study was 0.09 for SNP rs2772129, and the effect was in the same direction as the discovery sample. All other p values were greater than 0.33. The estimates for the variance explained by all SNPs using GCTA were somewhat higher in our samples than in the sample analyzed by Trzaskowski *et al.*,[12] in which the GCTA estimates varied between 1% and 19%. These investigators analyzed 4 anxiety dimensions (negative affect, negative cognition, fear, and social anxiety) and a general anxiety composite score consisting of the sum of the standardized scores of the 4 dimensions. The second highest GCTA estimate (0.16) was for the composite score. This could indicate that common SNPs explain more variance in a broader defined phenotype, as a narrower phenotype might be influenced by fewer SNPs.

To conclude, this study shows that a phenotype such as INT at age 3 years is genetically similar to adult phenotypes, that is, it is a polygenic disorder that is influenced by a large number of SNPs, each with a small effect. This signifies that with large enough samples, it is possible to detect genetic variants influencing preschool INT. This is even more important, given the overlap in results with GWA studies for other psychiatric disorders, indicating that these genetic variants also increase the risk for later psychiatric disorders. &

Accepted December 31, 2013.

This article was reviewed under and accepted by ad hoc editor James F. Leckman, MD.

Dr. Benke is with the Johns Hopkins Bloomberg School of Public Health, Baltimore, USA and the Samuel Lunenfeld Research Institute, University of Toronto, Toronto, Canada. Mr. Nivard is with VU University Amsterdam, The Netherlands and the Neuroscience Campus Amsterdam, VU University. Dr. Velders is with Erasmus Medical Center, Rotterdam, The Netherlands and The Generation R

Study Group, Erasmus Medical Center. Mr. Walters is with the University of Notre Dame, Notre Dame, USA. Dr. Pappa is with the School of Pedagogical and Educational Sciences, Erasmus University Rotterdam, the Generation R Study Group, and Erasmus University Medical Center-Sophia Children's Hospital. Dr. Scheet is with the University of Texas / M. D. Anderson Cancer Center, USA. Mr. Xiao is with the University of Texas / M. D. Anderson Cancer Center. Mr. Ehli is with the Avera Institute for Human Genetics, Sioux Falls, SD, USA. Dr. Palmer is with the Samuel Lunenfeld Research Institute, University of Toronto, and the Ontario Institute for Cancer Research, Toronto, Canada. Dr. Whitehouse is with Telethon Institute for Child Health Research, Subiaco, Australia, The Centre for Child Health Research, The University of Western Australia, Perth and the School of Psychology, The University of Western Australia, Crawley. Dr. Verhulst is with Erasmus Medical Center. Dr. Jaddoe is with Erasmus Medical Center, and The Generation R Study Group. Dr. Rivadeneira is with Erasmus Medical Center. Dr. Groen-Blokhuis is with VU University Amsterdam and EMGO+ Institute for Health and Care Research, VU University Medical Center. Dr. van Beijsterveldt is with VU University Amsterdam. Dr. Davies is with the Avera Institute for Human Genetics. Dr. Hudziak is with the Vermont Center for Children, Youth, and Families, and the University of Vermont, College of Medicine, Vermont, USA. Dr. Lubke is with the University of Notre Dame and with VU University Amsterdam. Dr. Boomsma is with the Neuroscience Campus Amsterdam, VU University Amsterdam, and EMGO+ Institute for Health and Care Research, VU University Medical Center. Dr. Pennell is with the School of Women's and Infants' Health, The University of Western Australia. Dr. Tiemeier is with Erasmus Medical Center. Dr. Middeldorp is with the Neuroscience Campus Amsterdam, VU University Amsterdam, and GGZinGeest/ VU University Medical Center.

The Netherlands Twin Registry (NTR) study was supported by the American Recovery and Reinvestment Act (ARRA) project RC2 2MH08995; the Netherlands Organisation for Scientific Research Spinozapremie prize (NWO/SPI 56-464-14192); NWO Center for Medical Systems Biology (CMSB); Twin-family database for behavior genetics and genomics studies (NWO 480-04-004); Genetic and Family Influences on Adolescent Psychopathology and Wellness (NWO 463-06-001); the Netherlands Bioinformatics Centre (NBIC) Bioassist (NWO-NBIC/BioAssist/RK/2008.024); Biobanking and Biomolecular Resources Research Infrastructure (NWO 184.021.007); Genetic influences on stability and change in psychopathology from childhood to your adulthood (ZonMW 91210020); and Genetics of Mental Illness (European Research Council; ERC-230374). The Raine study was supported by National Health and Medical Research Council (NHMRC), the University of Western Australia (UWA), Curtin University, the UWA Faculty of Medicine, Dentistry and Health Sciences, the Raine Medical Research Foundation, the Telethon Institute for Child Health Research, and the Women's and Infants Research Foundation. DNA collection and genotyping was funded by the NHMRC (572613). Dr. Whitehouse receives funding from Career Development Fellowships from the NHMRC (1004065). The Generation R Study was funded by the Erasmus Medical Center Rotterdam; the Erasmus University Rotterdam; The Netherlands Organization for Health Research and Development (ZonMw10.000.1003); the Netherlands Organisation for Scientific Research; the Ministry of Health, Welfare, and Sport; and the Ministry of Youth and Families. The current study was supported by a grant from the Sophia Foundation for Scientific Research (SKZ Foundation grant no. 491) and a NWO ZonMW VIDI grant (017.106.370; H.T.).

Drs. Benke and Velders and Mr. Nivard contributed equally to this research.

We thank the participants in the Netherlands Twin Register. The authors are extremely grateful to all of the families who took part in the Raine Study and the whole Raine Study team, which includes the Cohort Manager, Data Manager and data collection team. We thank C.M. van Duijn, PhD, of the Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands, for the careful reading of the manuscript.

Disclosure: Dr. Verhulst has received remuneration as contributing author of the Achenbach System of Empirically Based Assessment (ASEBA). Dr. Hudziak has received grant or research funding from the National Institute of Mental Health (NIMH) and the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK). His primary appointment is with the University of Vermont. He has additional appointments with Erasmus University in Rotterdam, Netherlands, Washington University School of Medicine in St. Louis, Missouri, Dartmouth School of Medicine in Hanover, New Hampshire, and Avera Institute of Human Behavioral Genetics in Sioux Falls, South Dakota. He serves as the Associate Editor of *JAACAP*. Drs. Benke, Velders, Pappa, Scheet, Palmer, Whitehouse, Jaddoe, Rivadeneira, Groen-Blokhuis, van Beijsterveldt, Davies, Lubke, Boomsma, Pennell, Tiemeier, and Middeldorp, and Mssrs. Nivard, Walters, Ehli, and Xiao report no biomedical financial interests or potential conflicts of interest.

Correspondence to Kelly Suzanne Benke, PhD, The Johns Hopkins Bloomberg School of Public Health, Mental Health Department, 60 North Broadway, 803 Hampton House, Baltimore, MD 21205; e-mail: kbenke@jhsph.edu

http://dx.doi.org/10.1016/j.jaac.2013.12.028

REFERENCES

1. Wichstrom L, Berg-Nielsen TS, Angold A, Egger HL, Solheim E, Sveen TH. Prevalence of psychiatric disorders in preschoolers. J Child Psychol Psychiatry. 2012;53:695-705.
2. Mesman J, Koot HM. Early preschool predictors of preadolescent internalizing and externalizing DSM-IV diagnoses. J Am Acad Child Adolesc Psychiatry. 2001;40:1029-1036.
3. Beyer T, Postert C, Muller JM, Furniss T. Prognosis and continuity of child mental health problems from preschool to primary school: results of a four-year longitudinal study. Child Psychiatry Hum Dev. 2012;43:533-543.
4. Pihlakoski L, Sourander A, Aromaa M, Rautava P, Helenius H, Sillanpaa M. The continuity of psychopathology from early childhood to preadolescence: a prospective cohort study of 3-12-year-old children. Eur Child Adolesc Psychiatry. 2006;15:409-417.
5. Bartels M, van den Oord EJ, Hudziak JJ, Rietveld MJ, van Beijsterveldt CE, Boomsma DI. Genetic and environmental mechanisms underlying stability and change in problem behaviors at ages 3, 7, 10, and 12. Dev Psychol. 2004;40:852-867.
6. Eley TC, Bolton D, O'Connor TG, Perrin S, Smith P, Plomin R. A twin study of anxiety-related behaviours in pre-school children. J Child Psychol Psychiatry. 2003;44:945-960.
7. Saudino KJ, Carter AS, Purper-Ouakil D, Gorwood P. The etiology of behavioral problems and competencies in very young twins. J Abnorm Psychol. 2008;117:48-62.
8. Van Hulle CA, Lemery-Chalfant K, Goldsmith HH. Genetic and environmental influences on socio-emotional behavior in toddlers: an initial twin study of the infant-toddler social and emotional assessment. J Child Psychol Psychiatry. 2007;48:1014-1024.
9. Gregory AM, Eley TC, Plomin R. Exploring the association between anxiety and conduct problems in a large sample of twins aged 2-4. J Abnorm Child Psychol. 2004;32:111-122.
10. Hettema JM, Neale MC, Kendler KS. A review and meta-analysis of the genetic epidemiology of anxiety disorders. Am J Psychiatry. 2001;158:1568-1578.
11. Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: review and meta-analysis. Am J Psychiatry. 2000;157:1552-1562.
12. Trzaskowski M, Eley TC, Davis OS, *et al*. First genome-wide association study on anxiety-related behaviours in childhood. PLoS One. 2013;8:e58676.
13. Yang J, Benyamin B, McEvoy BP, *et al*. Common SNPs explain a large proportion of the heritability for human height. Nat Genet. 2010;42:565-569.

14. Reef J, van Meurs I, Verhulst FC, van der Ende J. Children's problems predict adults' DSM-IV disorders across 24 years. J Am Acad Child Adolesc Psychiatry. 2010;49:1117-1124.
15. Cannon M, Caspi A, Moffitt TE, *et al*. Evidence for early-childhood, pan-developmental impairment specific to schizophreniform disorder: results from a longitudinal birth cohort. Arch Gen Psychiatry. 2002;59:449-456.
16. Kim-Cohen J, Caspi A, Moffitt TE, Harrington H, Milne BJ, Poulton R. Prior juvenile diagnoses in adults with mental disorder: developmental follow-back of a prospective-longitudinal cohort. Arch Gen Psychiatry. 2003;60:709-717.
17. Souery D, Oswald P, Massat I, Bailer U, Bollen J, Demyttenaere K, *et al*. Clinical factors associated with treatment resistance in major depressive disorder: results from a European multicenter study. J Clin Psychiatry. 2007;68:1062-1070.
18. Jaddoe VW, Mackenbach JP, Moll HA, *et al*. The generation R study: design and cohort profile. Eur J Epidemiol. 2006;21:475-484.
19. Jaddoe VW, van Duijn CM, Franco OH, *et al*. The Generation R Study: design and cohort update 2012. Eur J Epidemiol. 2012;27:739-756.
20. Jaddoe VW, Bakker R, van Duijn CM, *et al*. The Generation R Study Biobank: a resource for epidemiological studies in children and their parents. Eur J Epidemiol. 2007;22:917-923.
21. Boomsma DI, de Geus EJ, Vink JM, *et al*. Netherlands Twin Register: from twins to twin families. Twin Res Hum Genet. 2006;9:849-857.
22. van Beijsterveldt CE, Groen-Blokhuis M, Hottenga JJ, *et al*. The Young Netherlands Twin Register (YNTR): longitudinal twin and family studies in over 70,000 children. Twin Res Hum Genet. 2013;16:252-267.
23. McKnight CM, Newnham JP, Stanley FJ, Mountain JA, Landau LI, Beilin LJ, *et al*. Birth of a cohort—the first 20 2012s of the Raine study. Med J Aust. 2012;197:608-610.
24. Newnham JP, Evans SF, Michael CA, Stanley FJ, Landau LI. Effects of frequent ultrasound during pregnancy: a randomised controlled trial. Lancet. 1993;342:887-891.
25. Scheet P, Ehli EA, Xiao X, *et al*. Twins, tissue, and time: an assessment of SNPs and CNVs. Twin Res Hum Genet. 2012;15:737-745.
26. International HapMap Consortium. A haplotype map of the human genome. Nature. 2005;437:1299-1320.
27. Li Y, Willer CJ, Ding J, Scheet P, Abecasis GR. MaCH: Using sequence and genotype data to estimate haplotypes and unobserved genotypes. Genet Epidemiol. 2010;34:816-834.
28. Li Y, Willer C, Sanna S, Abecasis G. Genotype imputation. Annu Rev Genomics Hum Genet. 2009;10:387-406.
29. Purcell S, Neale B, Todd-Brown K, *et al*. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007;81:559-575.
30. So HC, Li M, Sham PC. Uncovering the total heritability explained by all true susceptibility variants in a genome-wide association study. Genet Epidemiol. 2011;35:447-456.
31. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics. 2010;26:2190-2191.
32. Bosker FJ, Hartman CA, Nolte IM, *et al*. Poor replication of candidate genes for major depressive disorder using genome-wide association data. Mol Psychiatry. 2011;16:516-532.
33. Visscher PM, Brown MA, McCarthy MI, Yang J. Five years of GWAS discovery. Am J Hum Genet. 2012;90:7-24.
34. Lee SH, Wray NR, Goddard ME, Visscher PM. Estimating missing heritability for disease from genome-wide association studies. Am J Hum Genet. 2011;88:294-305.
35. Lee SH, DeCandia TR, Ripke S, Yang J, *et al*; Schizophrenia Psychiatric Genome-Wide Association Study Consortium (PGC-SCZ), International Schizophrenia Consortium (ISC). Estimating the proportion of variation in susceptibility to schizophrenia captured by common SNPs. Nat Genet. 2012;44:247-250.
36. Demirkan A, Penninx BW, Hek K, *et al*. Genetic risk profiles for depression and anxiety in adult and elderly cohorts. Mol Psychiatry. 2011;16:773-783.
37. Lubke GH, Hottenga JJ, Walters R, *et al*. Estimating the genetic variance of major depressive disorder due to all single nucleotide polymorphisms. Biol Psychiatry. 2012;72:707-709.
38. Purcell SM, Wray NR, Stone JL, Visscher PM, O'Donovan MC, *et al*; International Schizophrenia Consortium. Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. Nature. 2009;460:748-752.
39. Smoller JW, Craddock N, Kendler K, Lee PH, Neale BM, *et al*; Cross-Disorder Group of the Psychiatric Genomics Consortium. Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. Lancet. 2013;381:1371-1379.
40. Williams NM, Zaharieva I, Martin A, *et al*. Rare chromosomal deletions and duplications in attention-deficit hyperactivity disorder: a genome-wide analysis. Lancet. 2010;376:1401-1408.
41. Hettema JM, Neale MC, Myers JM, Prescott CA, Kendler KS. A population-based twin study of the relationship between neuroticism and internalizing disorders. Am J Psychiatry. 2006;163:857-864.
42. Franic S, Dolan CV, Borsboom D, Hudziak JJ, Van Beijsterveldt CEM, Boomsma DI. Can genetics help psychometrics? Improving dimensionality assessment through genetic factor modeling. Psychol Methods. 2013;18:406-433.
43. Argyropoulos SV, Landau S, Kalidindi S, *et al*. Twins discordant for schizophrenia: psychopathology of the non-schizophrenic cotwins. Acta Psychiatr Scand. 2008;118:214-219.
44. McGuffin P, Rijsdijk F, Andrew M, Sham P, Katz R, Cardno A. The heritability of bipolar affective disorder and the genetic relationship to unipolar depression. Arch Gen Psychiatry. 2003;60:497-502.
45. Kan K, Dolan CV, Nivard MG, *et al*. Genetic and environmental stability in attention problems across the lifespan: evidence from the Netherlands twin register. J Am Acad Child Adolesc Psychiatry. 2013;52:12-25.
46. Purcell S, Cherny SS, Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics. 2003;19:149-150.
47. Lango Allen H, Estrada K, Lettre G, *et al*. Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature. 2010;467:832-838.
48. ENCODE Project Consortium. The ENCODE (ENCyclopedia of DNA elements) project. Science. 2004;306:636-640.
49. Birney E, Stamatoyannopoulos JA, Dutta A, *et al*; ENCODE Project Consortium. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature. 2007;447:799-816.
50. Dunham I, Kundaje A, Aldred SF, *et al*; ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012;489:57-74.
51. Malide D, Seidah NG, Chretien M, Bendayan M. Electron microscopic immunocytochemical evidence for the involvement of the convertases PC1 and PC2 in the processing of proinsulin in pancreatic beta-cells. J Histochem Cytochem. 1995;43:11-19.
52. Jonsson A, Isomaa B, Tuomi T, Eriksson JG, Groop L, Lyssenko V. Effect of a common variant of the PCSK2 gene on reduced insulin secretion. Diabetologia. 2012;55:3245-3251.
53. Leak TS, Keene KL, Langefeld CD, *et al*. Association of the proprotein convertase subtilisin/kexin-type 2 (PCSK2) gene with type 2 diabetes in an African American population. Mol Genet Metab. 2007;92:145-150.
54. Fujimaki T, Kato K, Yokoi K, *et al*. Association of genetic variants in SEMA3F, CLEC16A, LAMA3, and PCSK2 with myocardial infarction in Japanese individuals. Atherosclerosis. 2010;210:468-473.
55. Elks CE, Perry JR, Sulem P, *et al*. Thirty new loci for age at menarche identified by a meta-analysis of genome-wide association studies. Nat Genet. 2010;42:1077-1085.

SUPPLEMENT 1

Method

Each of the 3 cohorts participating in this genome-wide association (GWA) meta-analysis transformed the internalizing problem (INT) score by taking the square root. This was based on model diagnostics and results from data simulation. The INT score was right skewed so that a large density of scores was observed at 0 to low values, whereas an increasingly smaller number of scores was observed at the more extreme values. We were thus interested in determining the best way to handle this non-normal distribution.

We first simulated data to mimic the INT score, and then compared type I error, power, and bias for several modeling approaches. A score of 20 items with responses of 0, 1, or 2 and probability of 0.925, 0.05, and 0.025, respectively, were simulated; the items were correlated and summed to create a simulated score. The single nucleotide polymorphisms (SNP) frequency was set to 0.20, and the association of the summed score with the SNP was recorded. Analyses were repeated 1,000 times for different combinations of SNP effect size (and thus percent variation explained) and sample size. Four different models were tested: a generalized linear model (GLM) specifying a gamma distribution; a GLM specifying a Gaussian distribution; a GLM specifying a Gaussian distribution on the log transformation of the score; and a GLM specifying a Gaussian distribution on the square root transformation of the score. Simulation results for the 20 items score that was distributed similarly to our INT trait are provided in Table S3. Across a range of effect sizes and sample sizes, the square root transformation of the score generally resulted in the highest power.

In addition, model diagnostics were carried out in the Raine cohort among 1,737 participants with complete phenotype data, regardless of whether GWA data were available or not available, and plots of the residuals by fitted values were examined, as well as normal plots of the residuals. Model diagnostics suggested a considerably improved fit by transforming the INT score, particularly for the normalized plots of the residuals (data not shown). Between the natural log, log based 10 and square root transformations, the square root was judged to be the best fit in the Raine data. Along with the results from the simulation above, these findings justified our decision to perform the GWA using a square root transformation of the INT score.

To perform the phasing and imputation steps, Generation R and Raine studies used MaCH[1,2] and NTR used Beagle.[3] All positions reflect build 36 locations.

SUPPLEMENTAL REFERENCES

1. Li Y, Willer C, Sanna S, Abecasis G. Genotype imputation. Annu Rev Genomics Hum Genet. 2009;10:387-406.
2. Li Y, Willer CJ, Ding J, Scheet P, Abecasis GR. MaCH: Using sequence and genotype data to estimate haplotypes and unobserved genotypes. Genet Epidemiol. 2010;34:816-834.
3. Browning BL, Browning SR. A unified approach to genotype imputation and haplotype-phase inference for large data sets of trios and unrelated individuals. Am J Hum Genet. 2009;84:210-223.

FIGURE S1 Quantile–quantile plots for the association of genetic variants with internalizing problems (INT). Note: Plots are shown for each cohort separately and for the results from the meta-analysis of all cohorts combined. Gray-shaded areas represent 95% CI, and white lines represent equality between observed and expected −log10 (*p* values). NTR = Netherlands Twin Registry.

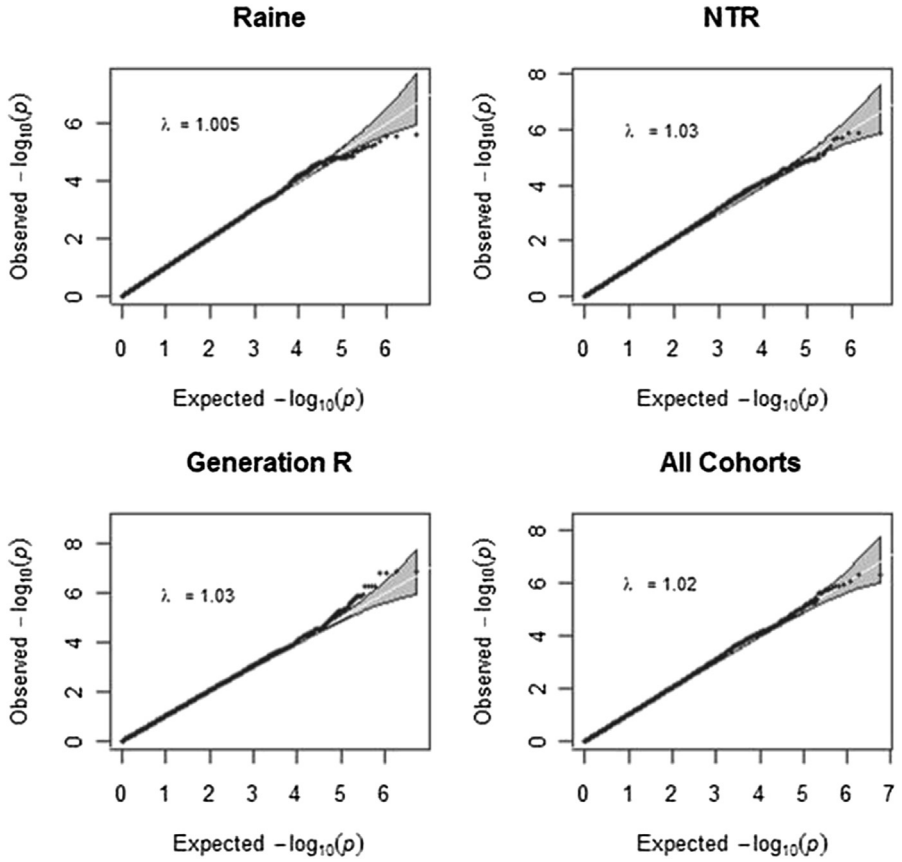


FIGURE S2 Manhattan plots for the genome-wide association analyses with internalizing problems (INT). Note: Plots are shown for each cohort separately. Chromosome is displayed on the x-axis and the association statistic, expressed as −log10(*p* value), is given on y axis. Points that fall within the black (−log10(5×10^{-8}) and gray lines (log10(1×10^{-5}) are suggestive but do not reach genome-wide significance. Pink points denote chr9 and chr20 single nucleotide polymorphisms (SNPs) that were of interest after meta-analysis. NTR = Netherlands Twin Registry.

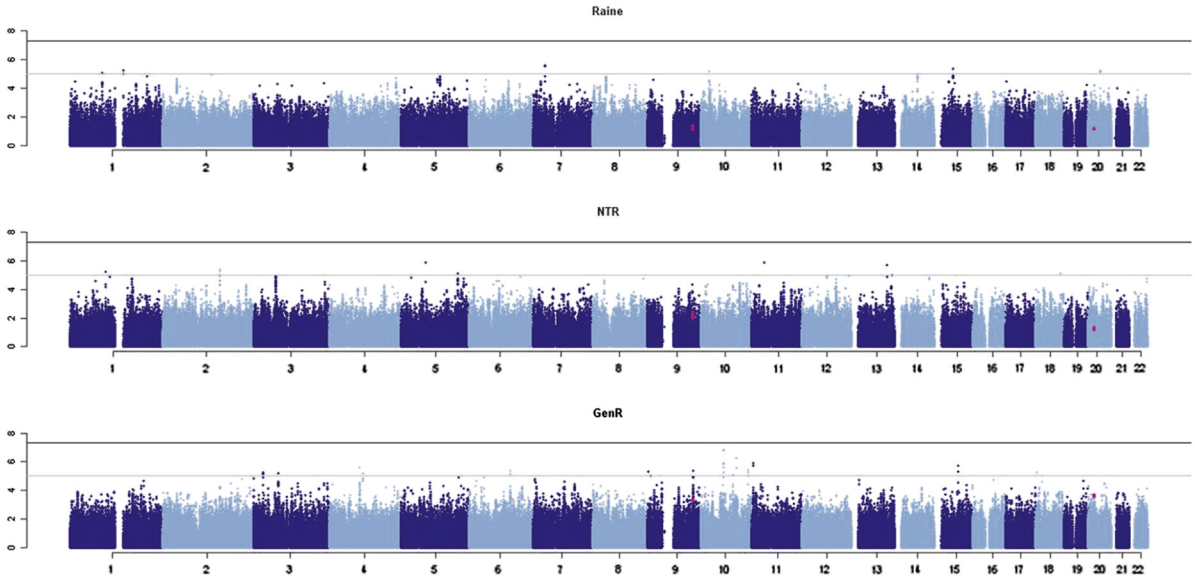


FIGURE S3 (A and B) Regional plots for the 2 regions that show suggestive signals. Note: *p* values reflect meta-analysis results across Netherlands Twin Registry (NTR), Generation R, and Raine cohorts. (A) Findings from chr9q33.1, an intergenic region that is not correlated with nearby genes. (B) Findings from chr20p12.1, which is in the PCSK2 gene. Plots were made using LocusZoom (https://statgen.sph.umich.edu/locuszoom). SNP = single nucleotide polymorphisms.

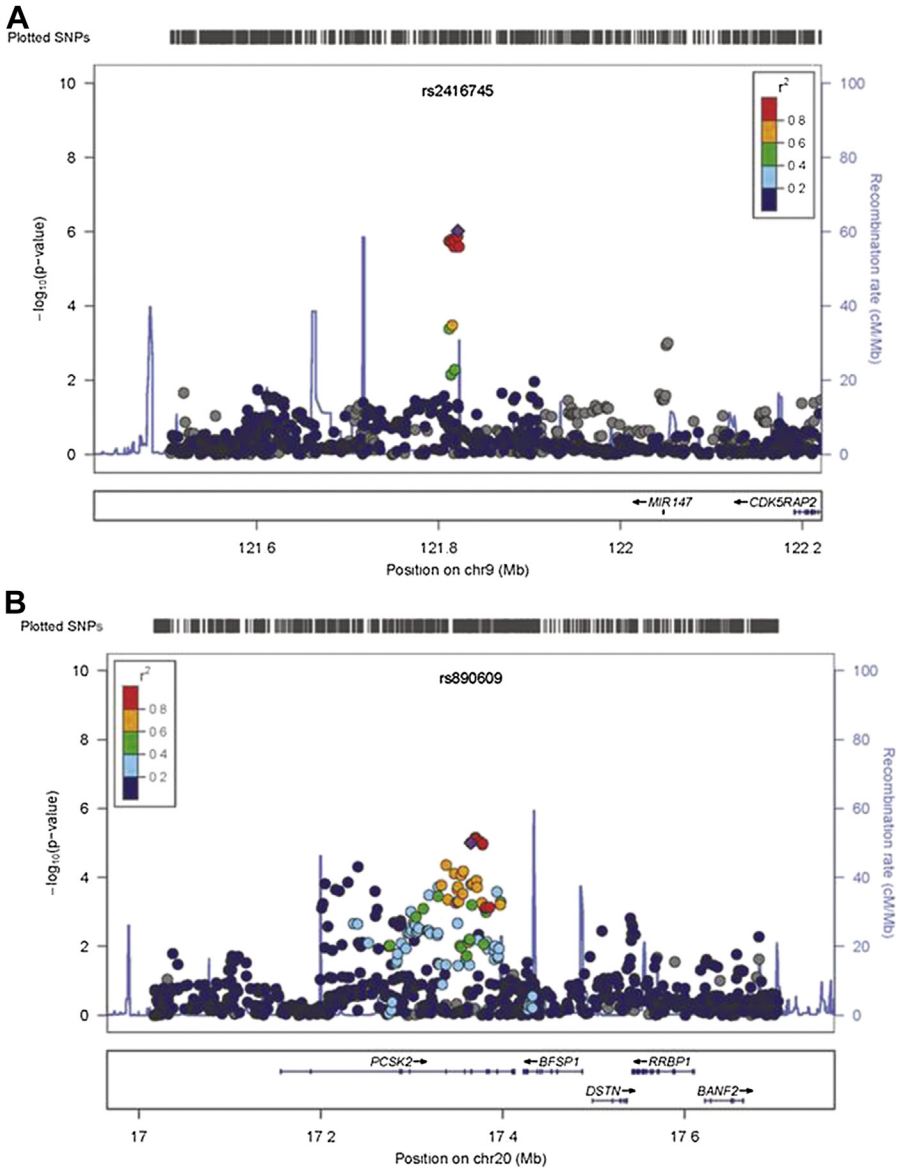


TABLE S1 Genotyping and Quality Control in Each Cohort

	Generation R	NTR	Raine
Genotyping Center	Genetic Laboratory, Department of Internal Medicine, Erasmus MC, Netherlands	Avera Institute for Human Genetics, Sioux Falls, South Dakota, USA	Centre for Applied Genomics, University of Toronto, Toronto, Canada
Genotyping platform	Illumina 610K Quad	Affymetrix 6.0	Illumina 660 Quad
Calling algorithm	Genomestudio 2009 V.1.1.9		Beadstudio
Imputation software	MACHqtl	BEAGLE	MACHqtl
Association software	MACH	PLINK	MACH
Reference panel for imputation	HapMap CEU r22 b36	HapMap CEU r22 b36	HapMap CEU r22 b36
Population stratification adjustments	2 principal components used as covariates	10 principal components used as covariates	2 principal components used as covariates
Filtering criteria			
Preimputation			
SNP level			
Call rate, %	95	95	95
HWE	<1x10-6	<0.00001	<5.7x10-7
Sample level			
% Missing	>2.5%	>1%	>3%
IBD	Π>0.1875	N/A	>3sd from HapMap CEU sample
Heterozygosity %	PLINK h<0.30	0.10< F > -0.10	< 4 sd from mean
Mendelian inhertance check	N/A	yes	N/A
Gender check	Yes	no	yes
Postimputation			
Quality metric (cutoff)	r2hat	info	r2hat
GC λ	1.030319	1.028017	1.004965
MAF	0.01	0.01	0.01

Note: GC = genetic complex; HapMap CEU = International HapMap Project; HWE = Hardy–Weinberg equilibrium; IBD = identity by descent; MAF = minor allele frequency; NTR = Netherlands Twin Registry; SNP = single nucleotide polymorphisms.

TABLE S2 Items on the Child Behavior Checklist Internalizing Problem Behavior Scale

Item	Subscale
Avoids looking others in the eye	Withdrawn
Acts too young for age	Withdrawn
Doesn't answer when people talk to him/her	Withdrawn
Seems unresponsive to affection	Withdrawn
Shows little affection toward people	Withdrawn
Shows little interest in things around him/her	Withdrawn
Refuses active games	Withdrawn
Withdrawn	Withdrawn
Disturbed by any change in routine	Emotionally reactive
Upset by new people or situations	Emotionally reactive
Sulks	Emotionally reactive
Worries	Emotionally reactive
Twitches	Emotionally reactive
Moody	Emotionally reactive
Whining	Emotionally reactive
Clings to adults or too dependent	Anxious/depressed
Gets too upset when separated from parents	Anxious/depressed
Self-conscious or easily embarrassed	Anxious/depressed
Too fearful or anxious	Anxious/depressed
Looks unhappy without good reason	Anxious/depressed
Unhappy, sad or depressed	Anxious/depressed
Feelings hurt	Anxious/depressed
Nervous	Anxious/depressed
Doesn't eat well	Somatic complaints
Aches	Somatic complaints
Can't stand things out of order	Somatic complaints
Constipated	Somatic complaints
Diarrhea	Somatic complaints
Headaches	Somatic complaints
Nausea	Somatic complaints
Painful bowel movements	Somatic complaints
Stomach aches	Somatic complaints
Too concerned with neatness/cleanliness	Somatic complaints
Vomits	Somatic complaints

TABLE S3 Power for Simulated Trait

N	Effect	No Transform, Gaussian	No Transform, Gamma	Log Transform, Gaussian	Square Root Transform, Gaussian
1,000	0.001	0.092	0.093	0.104	0.118
1,000	0.0025	0.186	0.187	0.181	0.217
1,000	0.01	0.618	0.617	0.505	0.642
1,000	0.05	0.999	0.999	0.976	0.999
1,500	0.001	0.137	0.137	0.143	0.176
1,500	0.0025	0.262	0.262	0.239	0.306
1,500	0.01	0.793	0.791	0.662	0.812
1,500	0.05	1	1	0.999	1
2,500	0.001	0.182	0.182	0.166	0.216
2,500	0.0025	0.407	0.407	0.348	0.452
2,500	0.01	0.933	0.933	0.877	0.971
2,500	0.05	1	1	1	1
5,000	0.001	0.36	0.36	0.304	0.371
5,000	0.0025	0.716	0.715	0.566	0.712
5,000	0.01	0.998	0.998	0.991	1
5,000	0.05	1	1	1	1